

FORM PTO-1390 (REV. 9-2001)		U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE		ATTORNEY'S DOCKET NUMBER CSI3-PT001	
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371				U.S. APPLICATION NO. (If known, see 37 CFR 1.5) 10/031520	
INTERNATIONAL APPLICATION NO. PCT/CN00/00205		INTERNATIONAL FILING DATE 19 July 2000		PRIORITY DATE CLAIMED 19 July 1999	
TITLE OF INVENTION PROTEOGLYCAN EXTRACTS OF ALGAE AND USES THEREOF					
APPLICANT(S) FOR DO/EO/US Qi et al.					
Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:					
<p>1. <input checked="" type="checkbox"/> This is a <b>FIRST</b> submission of items concerning a filing under 35 U.S.C. 371.</p> <p>2. <input type="checkbox"/> This is a <b>SECOND</b> or <b>SUBSEQUENT</b> submission of items concerning a filing under 35 U.S.C. 371.</p> <p>3. <input type="checkbox"/> This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include items (5), (6), (9) and (21) indicated below.</p> <p>4. <input type="checkbox"/> The US has been elected by the expiration of 19 months from the priority date (Article 31).</p> <p>5. <input checked="" type="checkbox"/> A copy of the International Application as filed (35 U.S.C. 371(c)(2))</p> <p style="margin-left: 20px;">a. <input type="checkbox"/> is attached hereto (required only if not communicated by the International Bureau).</p> <p style="margin-left: 20px;">b. <input checked="" type="checkbox"/> has been communicated by the International Bureau.</p> <p style="margin-left: 20px;">c. <input type="checkbox"/> is not required, as the application was filed in the United States Receiving Office (RO/US).</p> <p>6. <input checked="" type="checkbox"/> An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).</p> <p style="margin-left: 20px;">a. <input checked="" type="checkbox"/> is attached hereto.</p> <p style="margin-left: 20px;">b. <input type="checkbox"/> has been previously submitted under 35 U.S.C. 154(d)(4).</p> <p>7. <input checked="" type="checkbox"/> Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))</p> <p style="margin-left: 20px;">a. <input type="checkbox"/> are attached hereto (required only if not communicated by the International Bureau).</p> <p style="margin-left: 20px;">b. <input type="checkbox"/> have been communicated by the International Bureau.</p> <p style="margin-left: 20px;">c. <input type="checkbox"/> have not been made; however, the time limit for making such amendments has NOT expired.</p> <p style="margin-left: 20px;">d. <input checked="" type="checkbox"/> have not been made and will not be made.</p> <p>8. <input type="checkbox"/> An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371 (c)(3)).</p> <p>9. <input checked="" type="checkbox"/> An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).</p> <p>10. <input type="checkbox"/> An English language translation of the annexes of the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).</p> <p><b>Items 11 to 20 below concern document(s) or information included:</b></p> <p>11. <input type="checkbox"/> An Information Disclosure Statement under 37 CFR 1.97 and 1.98.</p> <p>12. <input type="checkbox"/> An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.</p> <p>13. <input type="checkbox"/> A <b>FIRST</b> preliminary amendment.</p> <p>14. <input type="checkbox"/> A <b>SECOND</b> or <b>SUBSEQUENT</b> preliminary amendment.</p> <p>15. <input type="checkbox"/> A substitute specification.</p> <p>16. <input type="checkbox"/> A change of power of attorney and/or address letter.</p> <p>17. <input type="checkbox"/> A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821 - 1.825.</p> <p>18. <input type="checkbox"/> A second copy of the published international application under 35 U.S.C. 154(d)(4).</p> <p>19. <input type="checkbox"/> A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).</p> <p>20. <input checked="" type="checkbox"/> Other items or information:</p> <p style="margin-left: 20px;">Copy of International Search Report (2 pgs.); Copy of International Preliminary Examination Report (6 pgs.); Application Data Sheet (2 pgs.); Drawings [4 sheets], in triplicate.</p>					

U.S. APPLICATION NO. <b>10/031520</b>		INTERNATIONAL APPLICATION NO. PCT/CN00/00205		ATTORNEY'S DOCKET NUMBER CSI3-PT001	
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21. <input checked="" type="checkbox"/> The following fees are submitted: <b>BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)):</b> Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO. .... <b>\$1040.00</b>  International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO ..... <b>\$890.00</b>  International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO ..... <b>\$740.00</b>  International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4) ..... <b>\$710.00</b>  International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4) ..... <b>\$100.00</b>  <b>ENTER APPROPRIATE BASIC FEE AMOUNT =</b>				<b>CALCULATIONS PTO USE ONLY</b>          	
Surcharge of <b>\$130.00</b> for furnishing the oath or declaration later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(e)).					
CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE	\$	
Total claims	56 - 20 =	36	x <b>\$18.00</b>	\$ 648.00	
Independent claims	8 - 3 =	5	x <b>\$84.00</b>	\$ 420.00	
MULTIPLE DEPENDENT CLAIM(S) (if applicable)				+ <b>\$280.00</b>	
<b>TOTAL OF ABOVE CALCULATIONS =</b>				\$ 2,108.00	
<input checked="" type="checkbox"/> Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by 1/2.				+	
<b>SUBTOTAL =</b>				\$ 1,054.00	
Processing fee of <b>\$130.00</b> for furnishing the English translation later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(f)).					
<b>TOTAL NATIONAL FEE =</b>				\$ 1,054.00	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). <b>\$40.00</b> per property +					
<b>TOTAL FEES ENCLOSED =</b>				\$ 1,054.00	
				<b>Amount to be refunded:</b>	\$
				<b>charged:</b>	\$

a. ☒ A check in the amount of \$ 1,054.00 to cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account No. \_\_\_\_\_ in the amount of \$ \_\_\_\_\_ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 22-0493. A duplicate copy of this sheet is enclosed.

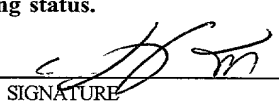
d. ☐ Fees are to be charged to a credit card. **WARNING:** Information on this form may become public. **Credit card information should not be included on this form.** Provide credit card information and authorization on PTO-2038.

**NOTE:** Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137 (a) or (b)) must be filed and granted to restore the application to pending status.

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4/PRTS

10/031520

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531 Rec'd

17 JAN 2002

## Proteoglycan Extracts of Algae and Uses thereof

### Field of the Invention

The present invention relates to an extract from algal organisms and its uses, especially to a proteoglycan extract of algae and uses thereof.

### Technical Background of the Invention

Since 70's, researchers have paid much more attention to studies on algal organisms, especially on blue-green algae. These studies are mainly concentrated on nutritional values and toxicity of the organisms. Of these, spirulina has been considered to be an excellent food resource of human in the future as early as in the Food and Agricultural Organization of the United Nations Conference held in 1974.

The properties of various extracts of algal organisms have been studied since 80's to see whether extracts of algal organisms can be used as potential pharmaceuticals. Of the studies, extracts of blue-green algae, in particular extracts of spirulina have been considered great significance.

Japanese patent application 58-12832 discloses a proteoglycan extracted from microalgae and spirulina inhibiting the growth of leukemia cells. However, no other activities of such a proteoglycan have been disclosed in this application. Although a process for extraction of the proteoglycan from spirulina disclosed therein, the process fails to involve the step of breaking the cellular wall of spirulina. Therefore, the process in this Japanese patent application is expected to be only applied in the laboratory, rather than in the industry with a large scale.

This invention is brought forward based on the inventors' studies on the extraction of proteoglycan of algae and therapeutic activities of the proteoglycan extract of algae.

### Summary of the Invention

One object of the present invention is to provide an anticancer composition comprising a therapeutically effective amount of proteoglycan extracts of algae and/or a pharmaceutically acceptable carrier.

Another object of the present invention is to provide a hemogram-improving composition comprising a therapeutically effective amount of proteoglycan extracts of algae and/or a pharmaceutically acceptable carrier.

Another object of the present invention is to provide an anti-irradiation composition comprising a therapeutically effective amount of proteoglycan extracts of algae and/or a pharmaceutically acceptable carrier.

Another object of the present invention is to provide a DNA-repairing composition comprising a therapeutically effective amount of proteoglycan extracts of algae and/or a pharmaceutically acceptable carrier.

Another object of the invention is to provide an antiviral composition comprising a therapeutically effective amount of proteoglycan extracts of algae and/or a pharmaceutically acceptable carrier.

Another object of the invention is to provide an immunoenhancing composition

comprising a therapeutically effective amount of proteoglycan extracts of algae and/or a pharmaceutically acceptable carrier.

Further another object of the invention is to provide a dendrite-like-cell-activating composition comprising a therapeutically effective amount of proteoglycan extracts of algae and/or a pharmaceutically acceptable carrier.

Still another object of the invention is to provide a process for preparing a proteoglycan extract of algae, including the following steps of:

- a. dissolving dry powder of blue-green algae in 5-20 times water by weight, and conducting cellular walls-breaking;
- b. heating a solution obtained from step a) at 60°-100°C, and cooling to separate a liquid phase from the solution;
- c. adjusting pH of the liquid phase to less than 7, and filtering; and
- d. adjusting the filter to pH 7, concentrating, and drying if necessary.

### Brief Description of the Drawings

**Figure 1** shows the effect of the hemogram in mice of a hemogram-improving composition of this invention;

**Figure 2** shows the effect of a proteoglycan extract of algae of this invention inhibiting Topo I-mediated supercoiled DNA relaxation;

**Figure 3** graphically shows the effect of a proteoglycan extract of algae of this invention inhibiting Topo II-mediated kDNA decatenation

**Figure 4** shows an agarose gel electrophoresis assay of a proteoglycan extract of algae of this invention inducing human HL-60 cell apoptosis;

**Figure 5** graphically shows a relationship between a concentration of a proteoglycan extract of algae of this invention and the percentage of apoptosis cell numbers;

**Figure 6** graphically shows the effect of different compositions according to the invention on a cell of bone marrow in mice;

**Figure 7** shows DNA contents of bone marrow in mice under different compositions in accordance with the invention;

**Figure 8** shows the effect of different doses of compositions according to the invention on  $\tau$  protein content in mice; and

**Figure 9** graphically shows the effect of a composition according to the invention on T lymphocytes.

### Detailed Description of the Invention

This invention provides a pharmaceutical composition comprising a proteoglycan extract of algae and/or a pharmaceutically acceptable carrier. In the composition of the invention, the amount of the proteoglycan extract of algae used is therapeutically effective. Since algal organisms have no by-effect on human being, the proteoglycan extract of algae used in the invention does in general not need to further be purified. However, the extract can be further processed to get a product of a higher purity, if necessary.

The amount of proteoglycan extract of algae used in the invention, which is

generally named with the term "therapeutically effective amount" in this application, can readily be determined by those skilled in the art. It depends on the circumstances of a patient, such as body weight, age, disease condition of the patient and the like. As stated before, the proteoglycan extract of algae used in the invention has no by-effect, it may directly be administered to patients. In this case, the composition comprises no pharmaceutically acceptable carriers. In case the composition contains a pharmaceutically acceptable carrier, the extract and the carrier can be mixed according to a conventional process in this field to obtain a desired preparation. Generally, the amount of the active component in the composition may be 0.1%-99.9% by weight.

Pharmaceutically acceptable carriers used in the invention include those conventionally used in the art, such as solvents, excipients and disintegrating agents. Pharmaceutical composition may be prepared as any conventional forms including oral solutions, capsules, troches, pills, powders, granules, syrups, suppositories, etc.

Powders of algae are preferably used as raw materials in the invention for extracting proteoglycan of algae. Cellular wall-breaking in step a) may be conducted in any conventional manners in the art, such as a supersonic treatment, a quickly stirring, an osmotic-pressure-changing lysis, or an enzymolysis.

To remove the cultures and impurities on the surface of algal powders, the surface of the powders may firstly be washed with little water. Generally, the time for heating in step b) of the invention is from 0.5h to 2h, preferably 1h, and the temperature therefor is in the range of 60°C-100°C, preferably 80°C-95°C, and more preferably at 95°C.

The amount of water used in the process of invention may be as 8-15 times by weight as that of algal powders used, preferably as 10 times by weight.

In step c), the solution is first adjusted to pH < 7, preferably to pH 2.0-4.5, and more preferably to pH 3.8-4.2. Conventional acids / bases such as a HCl or H<sub>2</sub>SO<sub>4</sub> solution and a Na<sub>2</sub>CO<sub>3</sub> or NaHCO<sub>3</sub> solution, may be used to adjust the pH value in this step.

In this invention, methods for separation between a solid phase and a liquid phase are those conventionally used in the art, such as filtration under a reduced pressure, molecular sieve filtration, centrifugation and the like. Because of the treatment of breaking cellular walls involved in this invention, proteoglycan extracts of algae may be obtained in a higher yield.

Proteoglycan extracts of algae obtained by the process of the invention have activities of anticancer, anti-irradiation, immunoenhancing, DNA-repairing, dendrite-like-cell-activating, hemopoietic function-improving and the like.

This invention will further be described with the following examples.

#### **Preparation of Proteoglycan Extracts of Algae**

##### **Example 1**

3kg of Spirulina powder was dissolved in 3L of water, and then filtered. To a

solution was added 30L water and stirred rapidly. Resultant solution was heated at 88°C for 1h. After cooling, the solution was filtered under a pressure. Then, the filtrate was adjusted to pH3.8 with a dilute solution of HCl and kept overnight. After centrifugation, the supernatant was adjusted to pH7 with a solution of Na<sub>2</sub>CO<sub>3</sub>. Resultant precipitates were spray-dried to obtain a crude extract of spirulina 0.599kg, which contains 72.3% of proteoglycan.

#### Example 2

3kg of Spirulina powder was dissolved in 24L of water, stirred and heated at 90°C for 1h. After cooling, the resultant was filtered under a reduced pressure. The filtrate was adjusted pH4.2 with HCl and kept overnight. After filtration, the filtrate was adjusted to pH7 with a solution of NaHCO<sub>3</sub>. Resultant precipitates were dried to obtain a crude proteoglycan extract of Spirulina 0.549kg, containing 71.2% of proteoglycan.

#### Biological Assays

##### 1. Anti-irradiation Activity

150 C-57 mice (18-22g) purchased from the Animal Center of Chinese University of Medical Sciences were used. Of them, 120 mice were irradiated with <sup>60</sup>Co-γ, at 600 rad, 8.64rad/min.

The irradiated mice were randomly divided into a control group, a lower dose group, a middle dose group, and a higher dose group, each with 30 mice, while 30 not-irradiated mice were used as a blank control group. Mice in the lower, middle, higher dose groups were administrated with the proteoglycan extract of spirulina obtained in Example 1 at a dose of 10mg/kg/d, 20mg/kg/d, 40mg/kg/d, respectively, whereas mice in the blank and control groups were not administrated with the extract. In ten days, survival rate of the mice in each group was shown in the following table.

Days after irradiation Groups	0	3	6	10	Survival rate (%) in 10 days
Higher Dose	30	30	26	22	73
Middle Dose	30	30	26	19	63
Lower Dose	30	30	26	10	33
Control	30	6	6	3	10
Blank	30	30	30	30	100

The result showed that survival rate of the mice in the administrated group was significantly higher than in the control group, proving that proteoglycan extract of spirulina possesses potent anti-irradiation activity, especially at a middle or higher dose.

2. Improving hemopoietic function and hemogram and enhancing the number of blood platelet

### Experiment 1. Hemogram Examination

C-57 mice (18-22g) purchased from the Animal Center of the Chinese University of Medical Sciences were used. One hundred and twenty mice were irradiated with  $^{60}\text{Co-}\gamma$ , at 600 rad, 8.64rad/min.

The irradiated mice were randomly divided into a control group, a positive control group, a lower dose group, a middle dose group, and a higher dose group, while 30 not-irradiated mice were used as a blank group. Mice in the positive group were administrated with shengxuewan (Tianjin Darentang Pharmaceutical Factory, China) at 6000mg/kg/d (as 20 times as an adult usually administrated). Mice in the lower, middle and higher dose groups were administrated with a proteoglycan extract of spirulina obtained in Example 1 at 10mg/kg/d, 20mg/kg/d, 40mg/kg/d, respectively, whereas mice in the blank and control groups were not administrated with the extract. Blood samples were taken from the tail vein of mice on the first day, the third day, the sixth day, respectively, after irradiation, and examined by a conventional hemogram-examining method.

The results were shown as follows:

Groups	Days after irradiation		
	First day (n=10)	Third day (n=10)	Sixth day (n=7)
Higher dose group	$120 \pm 10.62$ ( $<0.01$ )	$89.3 \pm 3.59$ ( $<0.01$ )	$163.7 \pm 22.1$ ( $<0.01$ )
Middle dose group	$70.6 \pm 7.74$ ( $<0.3$ )	$78.2 \pm 13.3$ ( $<0.05$ )	$109.3 \pm 8.03$ ( $<0.02$ )
Lower dose group	$86.1 \pm 9.79$ ( $<0.05$ )	$56.7 \pm 4.34$ ( $<0.3$ )	$89.0 \pm 9.76$ ( $>0.5$ )
Irradiation control Group	$57.2 \pm 6.02$	$47.0 \pm 3.82$	$85.6 \pm 4.5$
Blank control Group	$354.4 \pm 25.2$	$277.1 \pm 28.12$	$313.3 \pm 24054$

The data in table were expressed as  $\text{WBC} \pm \text{SE}$  and the total leukocyte number equals fifty times of the counting number while the data in brackets were P values compared with the irradiation control group and n is the animal number in each group.

The experiment showed that the number of leukocyte in treated groups is higher than that in the irradiation control group, particularly in the higher dose group ( $P < 0.01$ ). As seen in Figure 1, even the effect in the lower dose group also exceeded the control group.

### Experiment 2

One naval department processed a project under strong radiant conditions to make participants suffering radiant damage in the different degrees. After proteoglycan extracts of spirulina according to the invention were administrated, the number of leukocytes of the patients was increased, and the immunity thereof was enhanced. It showed that proteoglycan extracts of spirulina of the invention had

effects on increasing leukocytes.

### Experiment 3

30 persons whose level of leukocytes and platelets was relatively lower than that of the normal were tested. The proteoglycan extract of spirulina was orally administrated to the patients, twice a day and three tablets each time. After one month, 73 percent of the patients significantly increased in leukocytes and platelets, and the condition of the diet, rest and mental were improved. There was little effect on the other 26 percent of the patients since the number of their original leukocytes was not lower and still kept in the normal range.

### Experiment 4 Platelets Counting Assay

According to the requirements of the guidelines of novel drug on pre-clinical research, issued by the Chinese government, therapeutic effect of the proteoglycan extract of spirulina on beagle dogs was observed. The dogs were radiated with 6.5Gy<sup>60</sup>Co- $\gamma$  unevenly. The dogs were then administrated with an oral liquid for increasing leukocytes, produced by Zhengzhou Pharmaceutic Co. Ltd, as a positive control group. The dogs were administrated with capsules (360mg) made from the proteoglycan extracts according the invention as treated groups, three days before irradiation, and consecutively for 24 days. Results showed that there was little change to the specific volumes of leukocytes, granular leucocytes, red blood cells, haematoglobin and the blood cells of peripheral blood for the dogs in the positive control group. However, the amount of platelets for the dogs in each treated groups was obviously higher than that of the control group at the second and fourth week after irradiation ( $P < 0.01$ ). Furthermore, the recovery of the medullary macronucleocytes was also accelerated as well as the increase of the amount of macronucleocytes in marrow slides of the dogs in the treated groups. The transforming rate of the peripheral blood lymphocytes of the dogs in treated groups was also higher than that of an irradiation control (a blank control) group and the positive control group.

The above results showed that the proteoglycan extract of the invention can obviously recover the amount of the peripheral blood platelets and the transforming rate of the lymphocytes of beagle dogs after irradiation, and promote the proliferation of hematoblasts of the medullary macronuclear system thereof. Therefore, it is expected as a therapeutic agent to treat the tumor patients in radiotherapy and chemotherapy in a higher dose or the acute irradiated patients lacking the platelets or having decreased immune function.

### 3. Growth Inhibition of various Tumor Cell Lines

The proteoglycan extract according to the invention had distinct inhibitory effects on the proliferation of human leukemia cell lines such as U937, HL60 and P388 as well as human lung cancer A549, human hepatocellular hepatoma HepG2 and human gastric adenocarcinoma MKN-28, HCT116 in vitro. And it also exerted obvious



inhibition from the growth of mouse S180 sarcoma, B16 melanoma and human gastric adenocarcinoma MKN-28, SGC-7901 and human lung adenocarcinoma LAX83 nude mice xenografts in vivo.

## 5    **Expreiment 1. Growth Inhibition of various Tumors in vitro**

Tumor cell lines tested:

P388: mouse lymphoma

U937: human monocyte leukemia

10    HL60: human myeloid leukemia

K562: human erythroleukemia

A-549: human non-small cell lung cancer

SPC-A4: human lung adenocarcinoma

DMS-114: human small cell lung cancer

15    NCI-H23: human lung adenocarcinoma

SGC-7901: human gastric middle-differentiated adenocarcinoma

MKN-28: human gastric high-differentiated adenocarcinoma

HCT-116: human colon low-differentiated adenocarcinoma

Hep-G2: human hepatocellular hepatoma

20    MCF-7: human breast cancer

A-431: human dermal squamous cancer

The above cell lines are all kept and cultured in our lab.

## 25    **Measurements**

25    Suspended tumor cell lines, P-388, U-937, HL-60 and K-562 were measured by microculture tetrazolium (MTT) assay. Tumor cells in 90 $\mu$ l medium were seeded into each well of 96-well microculture plates at appropriate densities to maintain the cells in an exponential stage of growth during the experiment. Then, a solution of the proteoglycan extract obtained in the invention was added to each well, 10 $\mu$ l/well.

30    According to the pre-assay results, the proteoglycan extract of spirulina was designed for five concentrations and each was tested in triplicate wells. The blank having no cells and DMSO controls should be set for. After incubation at 37°C, 5%CO<sub>2</sub> for 48h, 20 $\mu$ l of 5mg/ml MTT solution was added to each well. After a further incubation was taken for 4h at 37°C, 50 $\mu$ l of a triplex solution (10%SDS-5% isobutanol-0.01mol/L

35    HCl) was added to each well and the plates were incubated overnight under CO<sub>2</sub>. The optical density (OD) was read on a plate reader at a wavelength of 570nm. The inhibitory rate of the cell proliferation was calculated by the following formula and the IC<sub>50</sub> value was calculated by the Logit method.

40    Other cell lines were measured by the Sulforhodamine B (SRB) assay. The adherent tumor cells were firstly allowed to attach for 24h then cultured by the same method as MTT assay. Briefly, after the attachment for 24h, the proteoglycan extract of spirulina at different concentrations was added to the well and the plates were cultured at 37°C for 72h. The mediums were removed and the cell were fixed by 100 $\mu$ l of 10% cold solution of trichloroacetic acid to each well, followed by

incubation at 4°C for 1h. The plates were washed with de-ionized water five times and dried in the air. The cells were then stained by 100µl of SRB (Sigma) solution in 1% acetic acid (V/V) per well for 15 min. After stained, the supernatant was removed and the plates were quickly washed five times with 1% acetic acid to remove the unbound dye and allowed to air dry. Bound dye was solubilized with 150µl of 10 mmol/L Tris base each well, then the optical density (OD) was read on a plate reader at a wavelength of 570 nm. The inhibitory rate and the IC50 value was calculated by the same method as the MTT assay.

$$\text{Growth Inhibitory Rate(\%)} = (\text{OD}_{\text{control}} - \text{OD}_{\text{treated}}) \times 100\% / \text{OD}_{\text{control}}$$

Table1 Inhibition rates (%) and IC50 values of the proteoglycan extract of spirulina on proliferation of tumor cell lines in vitro

Conc.(mg/ml)

Cell lines (mg/ml)	5	1.67	0.56	0.185	0.062	IC50
P-388	90.7	72.1	31.4	5.8	1.2	1.03
Hl-60	95.7	63.8	25.5	17.0	0.0	1.22
U-937	87.5	66.1	35.7	37.5	3.6	0.93
MKN-28	76.2	55.4	24.8	20.8	14.9	1.29
SGC-7901	48.3	6.2	2.1	3.4	0.0	----
A-431	0.0	0.0	0.0	1.8	4.6	----
SPC-4	41.2	23.5	26.5	9.8	0.0	---
	5	3.3	2.2	1.48	0.99	
A-549	92.4	86.4	50.0	30.3	54.5	2.00
NCI-H23	30.9	10.3	0.0	4.4	0.0	---
DMS-114	75.0	63.5	46.2	36.5	19.2	2.36
Hep-G2	85.9	87.5	75.0	39.8	23.4	1.61
K562	85.1	73.1	41.8	29.9	19.4	2.18
MCF-7	0.0	6.3	20.8	1.7	0.0	----
HCT-116	86.1	65.7	44.4	30.6	24.1	2.15

## Experiment 2 Inhibitory effect on DNA- topoisomerase and direct influence on DNA of proteoglycan extracts of spirulina

DNA topological structures of eukaryotic organisms are regulated by two classes of key enzymes, topoisomerase I (Topo I) and topoisomerase II (Topo II). Topo I can give rise to the break-up of a single-stranded DNA and plays an important role in DNA replication, transcription and chromosome organization although it isn't necessary for the survival of an eukaryotic cell. Topo II is necessary for a cell and can

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catalyze the break-up of a double-stranded DNA and plays an important role in DNA replication, transcription, recombination as well as forming the accurate chromosomal structure, chromosome segregation and condensation. In virtue of their critical functions and catalytic characterization, Topo I and Topo II have been identified as  
5 extensive targets of chemotherapeutic drugs in clinic.

DNA Topoisomerase inhibitors having DNA intercalation can bind DNA directly, but not break up DNA, while other kind of compounds as “molecular scissors” can cleave DNA directly. Both a DNA Topoisomerase inhibitor and molecular scissors make DNA metabolic turbulence, which gives rise to death of a cell.

## 10 **Methods:**

### 1. Effect of the proteoglycan extract of spirulina on Topo I activity

The proteoglycan extract of spirulina can inhibit relaxation of Topo I-mediated  
15 supercoiled PBR322.

### 2. Effect of the proteoglycan extract of spirulina on Topo II activity

The proteoglycan extract of spirulina can inhibit decatenation of Topo II-mediated kDNA.

20 Experimental results were seen in Figures 2 and 3.

The proteoglycan extract of spirulina inhibited the Topo I-mediated supercoiled PBR322 relaxation shown in Figure 2, in which

25 lane 1: PBR322 control; lane 2: 1 $\mu$  Topo I crude enzyme-extract solution; lane 3: 50  $\mu$ M OPT; lanes 4-10: 0.64, 3.2, 16, 80, 400, 2000, 10000 $\mu$ g/ml of DMSO-soluble components of the proteoglycan extract of spirulina; lanes 11-17: 0.64, 3.2, 16, 80, 400, 2000, 10000 $\mu$ g/ml of water-soluble components of the proteoglycan extract of spirulina.

30 The proteoglycan extract of spirulina can inhibit the Topo II-mediated kDNA decatenation as shown in Fig. 3, in which:

lane 1: kDNA control; lanes 2 and 10: 1 $\mu$  Topo II crude enzyme-extract solution; lane 3: 50mM VP16; lanes 4-9: 0.64, 3.2, 16, 80, 400, 2000 $\mu$ g/ml of DMSO-soluble components of the proteoglycan extract of spirulina; lanes 11-17: 0.64, 3.2, 16, 80, 400, 2000 $\mu$ g/ml of water-soluble components of the proteoglycan extract of spirulina;  
35 lane 18: 20% DMSO.

The results showed that two kinds of components of the proteoglycan extract of spirulina inhibited Topo I and Topo II activity both in the reaction of Topo I-mediated supercoiled PBR322 relaxation and in the reaction of the Topo II-mediated kDNA decatenation. But there was a relatively obvious difference between them. The water-soluble component had a higher inhibitory effect on Topo I, Topo II and showed a complete inhibitory effect on Topo I activity at 3.2 $\mu$ g/ml dose, and 16 $\mu$ g/ml for Topo II activity. The complete inhibitory dose of the DMSO-soluble component was 80 $\mu$ g/ml for Topo I and 2000 $\mu$ g/ml for Topo II, respectively.

40 These experiments indicated that the water-soluble component of the

proteoglycan extract of spirulina was main active components inhibiting topoisomerase, whose mechanism might be interacting with DNA firstly, inducing DNA conformation changed, then making the enzyme and substrate contact inefficiently and the enzyme activity decreased, even lost.

In summary, both the components of the proteoglycan extract of spirulina have obvious inhibitory effects on Topo I and Topo II. Furthermore, the water-soluble component can also induce the break-up of a double-stranded DNA directly.

### Experiment 3 Effect of the proteoglycan extract of spirulina on protein tyrosine kinase (PTK)

Protein tyrosine kinase (PTK) is a crucial factor in signaling transduction and is associated with the growth, proliferation and transformation of a cell. Many expression products of oncogenes are of PTK's activity, which is much higher in transformed cells than in normal cells. Therefore, it is hypothesized that the unexpected growth of tumor cells may be controlled by decreasing the PTK's activity.

Epidermal growth factor receptor (EGFR), a PTK of receptor type, is a single-stranded trans-membrane glycoprotein consisting of 1186 amino acids, whose molecular weight is 17kDa, and exists extensively on the epithelial membrane of the mammals. The C end of the EGFR can recognize and activate many cellular substrates after phosphorylated, then effect on metabolism, growth and carcinogenesis of a cell. Numerous researches have been indicated that the EGFR was overexpressed in many tumors and was tightly associated with tumor metastasis and poor prognosis. In our research, we used A431 cell containing the EGFR for picking up PTK, so as to observe the effect of the proteoglycan extract of spirulina on phosphorylation of PTK.

## RESULTS

### Results 1:

Conc.(mg/ml)	5.0	2.5	1.2	0.6	0.3
Inhibition rate(%)	100	100	100	67.9	65.0
Positive control: 2.5mM tyrphostin 25, inhibition rate:100%					

### Results 2:

Conc.(mg/ml)	1.5	0.75	0.4	0.2	0.1
Inhibition rate(%)	94.1	72.5	62.7	62.7	47.1
Positive control: 0.25mM tyrphostin 25, inhibition rate: 86.1%					

### Results 3:

Conc.(mg/ml)	1.2	0.6	0.3	0.15	0.07
Inhibition rate(%)	89	80.6	67.7	54.1	37.7
Positive control: 0.25mM tyrphostin 25, inhibition rate: 80.5%					

The results showed that the proteoglycan extract had a distinct inhibitory effect on the PTK's activity. It almost completely inhibited the tyrosine phosphorylation of PTK at a dose of 1.2mg/ml and had a 65-67% inhibitory rate at a dose of 0.3mg/ml, still had a 37.7% inhibitory rate at a lower dose of 0.07mg/ml.

The above experiments illustrated that the proteoglycan extract of spirulina could distinctly inhibit phosphorylation of the tyrosine residue of PTK. The possible mechanism is that it disrupts PTK-mediated signal transduction by inhibition of the kinase so as to prohibit the malignant growth of tumor cells.

#### **Experiment 4. Proteoglycan extract of spirulina inducing apoptosis in human leukemia HL-60 cells**

Apoptosis, regulated by a series of genes, is a programmed cell-death. Its regulatory turbulence was tightly associated with the malignant tumorigenesis. Now it is well-known that many anti-cancer drugs are able to induce apoptosis in tumor cells and the anti-cancer effect is related to the ability of inducing tumor cell apoptosis. Inducing cell apoptosis maybe a common pathway for anti-cancer drugs interfering tumor in different mechanisms, so cell apoptosis becomes one method of evaluating cure efficacy and inducing apoptosis of tumor cells is a target for curing tumor too.

### **Experiments and Results**

#### **Agarose Gel Electrophoresis**

When cell apoptosis occurred, endonuclease was active, DNA was cleaved between nucleosomes to form DNA fragments about 180-200bp or those of its multiple. Agarose gel electrophoresis could detect specific DNA ladders, see Figure 4. In Figure 4, numeral reference 1 represented a control group, numeral reference 2 represented a proteoglycan extract of spirulina group of 1mg/ml, numeral reference 3 represented a proteoglycan extract of spirulina group of 3mg/ml and numeral reference 4 represented a proteoglycan extract of spirulina group of 6mg/ml.

#### **Flow cytometry**

When cell apoptosis occurred, disrupt DNA fragments evaded from cells, and the amount of DNA in the cells decreased. Flow cytometry method can detect those cells before G1 phase (also called apoptosis cells), in which the amount of DNA is less than that of diploid cells. Results of the Experiment were shown in Figure 5.

The experiment demonstrated proteoglycan extracts of Spirulina had inhibit effects on a broad spectrum of human solid tumors and leukemia both in vitro and in vivo. It inhibited the growth of tumor cells as an inhibitory agent of the EGF-R protein tyrosine kinase by blocking signal pathways of proliferation of cells, and also induced a tumor to apoptosis as an inhibitory of topoisomerase by interfering with DNA replication, transcription and gene expression. Proteoglycan extracts of Spirulina had a little toxic, and could clinically be used in a long term. It had

significant anti-tumor effects and a clear target to be effected, so it should become a hopeful drug for treating cancers.

#### 4. Repairing DNA

##### Experiment 1 Bone marrow damage experiment

C-57 mice, body weight of 18-22 g, purchased from Animal Center of the Chinese Medical University. Mice were radiated with a dose of 600 rad  $^{60}\text{Co-}\gamma$ , 8.64 rad/min.

Groups	Cells/50mm <sup>2</sup> (Mean)	Percentage of Prematured cells (Mean)	Cell Mitosis
Control group	140	40	Yes
Radiated group	31	4	None
Lower dose group	72	30	Yes
Higher dose group	74	35	Yes
Positive control group	45	35	Yes

Mice after radiated were divided into groups of a radiated control, lower dose, middle dose, and higher dose groups. Mice in the control group had not been radiated. Mice in the control group and the radiated control group were treated without drugs, mice in the lower dose group were treated with Proteoglycan extracts of Spirulina at a dose of 10mg/kg/day, in middle dose group with Proteoglycan extracts of Spirulina at a dose of 20mg/kg/day, and in the higher dose group with Proteoglycan extracts of Spirulina at a dose of 40mg/kg/day. At the second day after radiation, 10 mice randomly selected from each group were autopsied for tissue slices. Slices were observed under a microscope, amplified with 750 times.

Experiment results shown in Figure 6 demonstrated that the damage of radiation to the mice was most significant in the radiated control group. In this group, bone marrow of the mice was of a network-like, cell proliferation thereof was disrupted, premature cell thereof only occupied 4%, and no mitosis was observed. Blood capillary wall in bone marrow was damaged to cause hemorrhage and form a "blood pool". The damage of the mice in the groups treated with drug was reduced.

##### Experiment 2 Determining the amount of bone marrow DNA

The mice were radiated and treated as above. At the sixth day after radiation, 10 mice randomly selected from each group were autopsied. An intact femur removing all soft tissues was washed with 10 ml solution of 0.005M  $\text{CaCl}_2$  to introduce all bone marrow into a centrifugal tube, kept at 4°C for 30 min, and centrifuged with 2500rpm for 15 min. Precipitate was fully mixed with 5 ml solution of 0.2 mol/l  $\text{HClO}_4$ , heated at 90°C for 15 min, and centrifuged after cooling. The value of OD of the

supernatant at 286nm was detected. DNA contents: 1OD=33  $\mu$  g/ml DNA.

Experimental results were listed as follows:

Group	Control group	Radiated control group	Lower dose group	Middle dose group	Higher dose group	Positive control group
DNA $\mu$ g/ml	49.04	2.01	2.71	8.94	6.34	3.56
	27.03	2.94	8.42	10.00	4.82	2.38
	27.92	1.58	7.19	7.79	6.14	4.79
	27.19		4.42		3.70	6.34
Mean	32.79	2.18	5.68	8.91	5.25	4.27
P			<0.05	<0.01	<0.01	<0.01

The results were also expressed as in Figure 7, which demonstrated: DNA content in the treated group was higher than that of the radiated control group. The middle dose, higher dose or positive control group, compared to the radiated control group, had a significantly difference ( $P<0.01$ ). It showed the extract according to the invention protected bone marrow cells, and had obviously repairing effect on DNA.

## 5. Antiviral experiment

### Clinical pathology:

Patient 1: XXX, male, middle age, a farmer of Daxing County, Beijing. He suffered viral hepatitis, and became cirrhosis later. After 10 year, he had serious ascitic fluid, and hemorrhage in digestive tract. After treated with a capsule made from proteoglycan extracts of Spirulina according to the invention twice daily, 1 capsule per time, there were less ascitic fluid and great hemorrhage after 60 days, there was little ascitic fluid after 90 days, and he could deal with his own daily life, and was recovered. In 1999, he did CT test in hospital, compared with CT result 10 years ago, the focus did not expand or get worse. According to pathologists, it was rare for a patient not to enlarge the focus suffering cirrhosis for 10 years.

Patient 2: XXX, a head of an elementary school in Beijing, had cirrhosis because he did not cure viral hepatitis well, followed with serious ascitic fluid and great hemorrhage in digestive tract and was to die. After treated with proteoglycan extracts of Spirulina, twice daily, one capsule per time, continued 60 days, there were less ascitic fluid obviously, and no great hemorrhage any more. After treated for 90 days, there were little ascitic fluid; after treated with drug for 180 days, he could deal with his own daily life, back to work again, and feel good. In 1999, he did CT test in 301 hospital, and find no focus. Pathologists believed there was evidence to consider that virus was inhibited.

## 6. Repairing mucous membrane damage

C-57 mice, body weight of 18-22 g, purchased from the Animal Center of Chinese Medical University. Mice were radiated with a dose of 600 rad  $^{60}\text{Co-}\gamma$ , 8.64 rad/min.

Mice after radiation were divided into groups of a radiated control, a lower dose, middle dose, and a higher dose. Mice in a blank control group were not radiated. Mice in the blank control group and a radiated control group were treated without drugs. Mice in the lower dose group were treated with proteoglycan extracts of Spirulina according to the invention at a dose of 10mg/kg/day, in the middle dose group at a dose of 20mg/kg/day, and in the high dose group at a dose of 40mg/kg/day. At the seventh and fourteenth day after radiation, 10 mice randomly selected from each group were autopsied, and made tissue slices for small intestine. The slices were observed under a microscope, photographed and counted.

Experiment data showed: for the mice in the control group, radiation generated great harm to epithelial cells of small intestine mucous membrane of the mice, caused epithelial cells of mucous membrane of the mince damaged, even disruption. This also made the stratum of the cells naked. However, the harm under radiation to epithelial cells of small intestine mucous membrane of the mice in the treated group was of a significant decrease, compared to the control group.

## 7. Enhancing immune system

### Experiment 1 Content of $\gamma$ globulin

C-57 mice, body weight of 18-22 g, purchased from Animal Center of Chinese Medical University, were radiated with a dose of 600 rad  $^{60}\text{Co}-\gamma$ , 8.64 rad/min.

Mice after radiation were divided into a radiated control group and a radiated treated group. Mice in a control group and a treated control group were not radiated. Blood harvested from mice's eye sockets was centrifuged and serum was taken therefrom. The serum was conducted a chromatographic analysis after dyeing. The content of  $\gamma$ -globulin in serum was calculated according to peak values.

The results were as follows:

Group	Mean of $\gamma$ globulin percentage( $\pm$ SD)
Control	10.07 $\pm$ 3.57
Treated control	12.84 $\pm$ 6.75
Radiated control	6.19 $\pm$ 4.96
Radiated treated	13.32 $\pm$ 6.73

The results are also expressed as in Figure 8, which demonstrated that the mice both in the radiated group and the not radiated group,  $\gamma$  globulin percentage of the mice in the treated group were higher than that of the corresponding control group. Since the content of  $\gamma$  globulin represents immune function of a body, the increase of  $\gamma$  globulin percentage showed improvement of immune function of a body.

### Experiment 2 T-lymphocyte Test

Mice, body weight of 18-22g, were randomly divided into a blank control group, positive control group, a higher dose group and a lower dose group, with 10 mice per group. The mice in the treated groups were injected with CTX at a dose of 10mg/kg/d,



and then treated with proteoglycan extracts of Spirulina via p.o., at the same dose as above. The mice in the positive control group were treated with CTX at a dose of 10mg/kg/d, and then treated with water via p.o. the mice in the control group only were treated with water via p.o. after the treatment was continuously taken for 10 days, the treatment was stopped for 2 days. Blood was harvested from eye sockets of the mice for lymphocyte slices. After incubation and dyeing, T-lymphocyte percentage was determined.

Results were shown as follows:

Groups	T-lymphocyte	P value
Control	28.0±2.55	
Positive control	25.6±2.51	
Higher dose	39.8±7.40	<0.01
Lower dose	33.2±6.30	<0.05

The results were also expressed as in Figure 9, which demonstrated that after the mice were injected with CTX, the content of T-lymphocyte of the mice decreased significantly. However, the content of T-lymphocyte of the mice in the treated group increased, especially in the higher dose group,  $P < 0.01$ . This showed that proteoglycan extracts of Spirulina according to the invention had function of enhancing immune function of a body and protection of bone marrow cells.

#### 8. Proteoglycan extracts of Spirulina on proliferation of CD43+ hemotapoietic stem/ancestor cells

##### Materials and Methods:

All cells were cultured with corresponding cell factors at 37°C in 15%FCS RPMI 1640 medium under a atmosphere of 5% CO<sub>2</sub>.

Umbilical core blood CD34+ hemotapoietic stem/ancestor cells

A sample, umbilical core blood came from People Hospital, Department of Gynaecology and Obstetrics, Beijing Medical University. Final concentration of heparin was 20U/ml. The sample was screened CD34+ cells within 5 hours after harvesting blood. Umbilical core blood was diluted with PBS. Erythrocyte was precipitated with 0.1% methylcellulose, and the supernatant was collected. Monocyte (MNC) was separated with lymphocyte separating medium. Using QBEND-10 as CD34 antibody and sheep anti-mouse IgG1 immune beads (Miltenyi Biotec Co. Germany). Marked cells were passed through separating column in a magnetic field., and un-marked cells is removed by elution. The column was removed from the magnetic field, and eluted under a pressure. CD34+ cells were collected and counted.

##### Cell factors

Cell factors and the manufactory thereof in CD34<sup>+</sup> cell culture were listed below:

# Cell factors in the culture and the sources thereof

Factors	Sources
FL	Immunex
Tpo	Glaxo
SCF	Sandos
IL-6	Sandos
IL-3	Sandos
G-CSF	Amgen
GM-CSF	Amgen

## Antibody

Mono-clone antibody used in a flow cytometry included CD34-FITC, HLA-DR-FITC, CD33-PE, CD1a-PE, CD42b-PE, came from Pharmingen Co.

## Detection of effects on proliferation and differentiation

of CD43+ hemotapoeitic stem/ancestor cells

CD43+ hemotapoeitic stem/ancestor cells were cultured in a medium consisting of IMDM, 15%FCS, 15%HS, 40ng/ml FL, 20ng/ml Tpo, 200ng/ml SCF, 10ng/ml IL-6, 10ng/ml IL-3 and 2X104u/mlG-CSF. CD34+ cells were incubated and cultured in a 24-cell plate, 3X10<sup>4</sup> cells/ml, 1ml/cell. 3 Cells, as a group, were treated with proteoglycan extracts of Spirulina at a dose of 0, 0.2, 0.4 and 0.8mg/ml, respectively. Cells were cultured at 37°C under 5% CO<sub>2</sub> and a saturated humidity, and added by cell factors every 48 hours. After cultured ten days, cells were harvested, and detected for the cell phenotype with the corresponding fluorentscent antibody.

## Experiment results:

In the presence of FL, TPO, SCF, IL-6, IL-3 and G-CSF, the proliferation of CD34+ cells showed a negative relation to concentrations of Proteoglycan extracts of Spirulina. That is, in the absence of Proteoglycan extracts of Spirulina, cell proliferation and differentiation were active, and cell numbers and differentiation rate were increased. After cultured for 10 days, most CD34+ cells differentiated into mature cells. With the increase of concentrations of Proteoglycan extracts of Spirulina, cell proliferation decreased, and cell deformation and anchoring cells were increased under a microscope. This demonstrated that Proteoglycan extracts of Spirulina could reverse the proliferation of CD34+ cells boosted by cell factors, and could decrease the differentiation rate of CD34+ cells. After the culture, the percentage of CD34+ cells and granular and nucleus ancestor cells in the treated groups were higher than those of the groups only treated by cell factors.

Treated with different concentrations of Proteoglycan extracts of Spirulina, CD34+ cells was detected by a flow cytometry after cultured for 10 days. Results were shown below:

Proteoglycan extracts of Spirulina concentration (mg/ml)	Cell percentage (%)				
	CD34+	CD34+CD33+	CD34+CD42b+	CD1a+	HLA-DR+
0	0.07	0.08	0	1.7	26.87
0.2	1.10	0.71	0.16	13.09	73.94
0.4	4.83	2.08	1.77	23.12	86.49
0.8	25.75	7.78	9.03	19.02	80.98

From the above table, when the concentration of proteoglycan extracts of Spirulina was increased, the content of CD34+ cells was accordingly increased significantly. So were the content of CD34+CD33+ cells which represent granular and nucleus ancestor cells and that of CD34+CD42b+ cells which represent monocyte ancestor cells. From the above table also, it showed the content of CD1a+ and HLA-DR+ that represent dendritic cells (DC) was significantly increased with the increase of concentrations of proteoglycan extracts of Spirulina. There were a lot of typical dendritic cells emerged in the culture. This indicated that proteoglycan extracts of Spirulina according to the invention had effects on inducing to differentiate CD34+ cells into dendritic cells, reaching a peak at a dose of 0.4mg/ml of the extracts. Proteoglycan extracts of Spirulina stimulating lymphocyte to secrete cell factors

The following table showed that Proteoglycan extracts of Spirulina with different dosages stimulated lymphocyte to secrete IL-2, IL-3, GM-CSF and IFN- $\gamma$ . It showed that Proteoglycan extracts of Spirulina had significant effects on stimulating lymphocyte to secrete GM-CSF, which were related to the dosage of the extracts used. With the increase of the dosage of proteoglycan extracts of Spirulina used, GM-CSF's secretion was correspondingly increased. When the concentration of proteoglycan extracts of Spirulina was 0.8mg/ml, GM-CSF secretion amount was 1.15 times as that of the untreated. However, stimulation effects of Proteoglycan extracts of Spirulina on secreting IL-2, IL-3 and IFN- $\gamma$  were not significant.

#### Proteoglycan extracts of Spirulina stimulating

Proteoglycan extracts of Spirulina (mg/ml)	human peripheral blood lymphocyte secrete cytokine			
	0	0.2	0.4	0.8
GM-CSF (pg/ml)	20.4	67.3	196.2	235.3
IL-2 (pg/ml)	0	1.2	0	0
IL-3 (pg/ml)	3.2	3.2	2.9	3.0
IFN- $\gamma$ (pg/ml)	13.8	12.5	12.8	13.0

#### Conclusion:

1. Proteoglycan extracts of Spirulina could negatively regulate proliferation of umbilic CD34<sup>+</sup> hemopoietic stem/ancestor cells in the presence of cell factors, and greatly slower the differentiation of CD34<sup>+</sup> cells. In the CD34<sup>+</sup> cells culture system, with the increase of concentrations of proteoglycan extracts of Spirulina, the content of CD34<sup>+</sup> hemopoietic stem/ancestor cells, CD34<sup>+</sup>CD33<sup>+</sup> myeloid ancestor cells and CD34<sup>+</sup>CD42b<sup>+</sup> macronucleus ancestor cells was much higher than that in a group to

which only cell factors were added. This revealed that proteoglycan extracts of Spirulina could partly inhibit the proliferation of CD34<sup>+</sup> by cell factors, slower the differentiation of hemopoietic stem/ancestor cells, make myeloid and macronucleus ancestor cells in a higher proportion, and keep hemopoiesis for a longer time. It was expected that in an animal experiment, the recruit of myelocytes and platelet by proteoglycan extracts of Spirulina would be later than that by cell factors, but the maintenance of myelocytes and platelet by them would be in evidence.

2. Dendritic cell (DC) is the most efficient antigen-presenting cell (APC), which plays an important role in immunoreaction of host. It can recognize the mutant cells, transfer signals to effector cells, and kill tumor cells at last. In vivo, DC is often too few to take functions. So how to increase the number of DC becomes one of the focuses in studying on antitumor immunotherapy and its clinic application. Although no DC induced factors such as GM-CSF, IL-4 or TNF- $\alpha$  are added to the culture system in the present invention, the proportion of CD1a<sup>+</sup> and HLA-DR<sup>+</sup> cells (important surface marker of DC) increases along with the increasing of concentrations of proteoglycan extracts of Spirulina. It shows that proteoglycan extracts of Spirulina can induce CD34<sup>+</sup> hemopoietic stem/ancestor cells differentiation into DC. When concentrations of proteoglycan extracts of Spirulina are at 0.4 mg/ml, the induced efficiency becomes the highest (the content of CD1a<sup>+</sup> and HLA-DR<sup>+</sup> cells increased respectively from that of 1.70% and 26.87% to 23.12% and 86.49% in the control group). It implies that proteoglycan extracts of Spirulina are of a potential value and have wide prospects in antitumor immunotherapy.

3. Proteoglycan extracts of Spirulina can remarkably stimulate lymphocyte to secrete GM-CSF, with a typical relationship with the dosage of the extracts. The secretion of GM-CSF increases obviously along with the increasing of concentrations of proteoglycan extracts of Spirulina (the secretion of GM-CSF increasing by 11.5 times with a concentration of 0.8 mg/ml). It reveals that proteoglycan extracts of Spirulina can improve the recovery of haematogenous function, and have wide effects on promoting hemopoietic cells proliferation. In addition, GM-CSF is one of the most important cell factors to promote DC proliferation, maturing and function, which is one of the possible mechanisms of DC formation induced by proteoglycan extracts of Spirulina according to the invention.

We claim:

1. An anticancer composition comprising a therapeutically effective amount of proteoglycan extract of algae and/or a pharmaceutically acceptable carrier, wherein the proteoglycan extract of algae is prepared by the following steps of :

- a) dissolving a dry powder of blue-green algae in 5-20 times water by weight, and conducting cellular walls-breaking;
- b) heating a solution obtained from step a) at 60°-100°C, and cooling to separate a liquid phase from the solution;
- c) adjusting pH of said liquid phase to less than 7, and filtering; and
- d) adjusting the filter to pH 7, concentrating, and drying if necessary.

2. The composition according to claim 1, wherein said blue-green algae is *Spirulina*.

3. The composition according to claim 1, wherein said water in step a) is used as 8-15 times as that of said dry powder.

4. The composition according to claim 3, wherein said water in step a) is used as 10 times as that of said dry powder.

5. The composition according to claim 1, wherein said step b) is conducted at a temperature of 80°C-95°C.

6. The composition according to claim 5, wherein said step b) is conducted at a temperature of 90°C.

7. The composition according to claim 1, wherein said liquid phase in step c) is adjusted to pH 3.8-4.2.

8. A hemogram-improving composition comprising a therapeutically effective amount of proteoglycan extract of algae and/or a pharmaceutically acceptable carrier, wherein the proteoglycan extract of algae is prepared by the following steps of:

- a) dissolving a dry powder of blue-green algae in 5-20 times water by weight, and conducting cellular walls-breaking;
- b) heating a solution obtained from step a) at 60°-100°C, and cooling to separate a liquid phase from the solution;
- c) adjusting pH of said liquid phase to less than 7, and filtering; and
- d) adjusting the filter to pH 7, concentrating, and drying if necessary.

9. The composition according to claim 8, wherein said blue-green algae is *Spirulina*.

10. The composition according to claim 8, wherein said water in step a) is used as 8-15 times as that of said dry powder.

11. The composition according to claim 10, wherein said water in step a) is used as 10 times as that of said dry powder.

12. The composition according to claim 8, wherein said step b) is conducted at a temperature of 80°C-95°C.

13. The composition according to claim 12, wherein said step b) is conducted at a temperature of 90°C.

14. The composition according to claim 8, wherein said liquid phase in step c) is

adjusted to pH 3.8-4.2.

15. An anti-irradiation composition comprising a therapeutically effective amount of proteoglycan extract of algae and/or a pharmaceutically acceptable carrier, wherein the proteoglycan extract of algae is prepared by the following steps of:

- a) dissolving a dry powder of blue-green algae in 5-20 times water by weight, and conducting cellular walls-breaking;
- b) heating a solution obtained from step a) at 60°-100°C, and cooling to separate a liquid phase from the solution;
- c) adjusting pH of said liquid phase to less than 7, and filtering; and
- d) adjusting the filter to pH 7, concentrating, and drying if necessary.

16. The composition according to claim 15, wherein said blue-green algae is *Spirulina*.

17. The composition according to claim 15, wherein said water in step a) is used as 8-15 times as that of said dry powder.

18. The composition according to claim 17, wherein said water in step a) is used as 10 times as that of said dry powder.

19. The composition according to claim 15, wherein said step b) is conducted at a temperature of 80°C-95°C.

20. The composition according to claim 19, wherein said step b) is conducted at a temperature of 90°C.

21. The composition according to claim 15, wherein said liquid phase in step c) is adjusted to pH 3.8-4.2.

22. A DNA-repairing composition comprising a therapeutically effective amount of proteoglycan extract of algae and/or a pharmaceutically acceptable carrier, wherein the proteoglycan extract of algae is prepared by the following steps of:

- a) dissolving a dry powder of blue-green algae in 5-20 times water by weight, and conducting cellular walls-breaking;
- b) heating a solution obtained from step a) at 60°-100°C, and cooling to separate a liquid phase from the solution;
- c) adjusting pH of said liquid phase to less than 7, and filtering; and
- d) adjusting the filter to pH 7, concentrating, and drying if necessary.

23. The composition according to claim 22, wherein said blue-green algae is *Spirulina*.

24. The composition according to claim 22, wherein said water in step a) is used as 8-15 times as that of said dry powder.

25. The composition according to claim 24, wherein said water in step a) is used as 10 times as that of said dry powder.

26. The composition according to claim 22, wherein said step b) is conducted at a temperature of 80°C-95° C.

27. The composition according to claim 26, wherein said step b) is conducted at a temperature of 90°C.

28. The composition according to claim 22, wherein said liquid phase in step c) is adjusted to pH 3.8-4.2.

29. An antiviral composition comprising a therapeutically effective amount of

proteoglycan extract of algae and/or a pharmaceutically acceptable carrier, wherein the proteoglycan extract of algae is prepared by the following steps of:

a) dissolving a dry powder of blue-green algae in 5-20 times water by weight, and conducting cellular walls-breaking;

5 c) heating a solution obtained from step a) at 60°-100°C, and cooling to separate a liquid phase from the solution;

c) adjusting pH of said liquid phase to less than 7, and filtering; and

d) adjusting the filter to pH 7, concentrating, and drying if necessary.

10 30. The composition according to claim 29, wherein said blue-green algae is *Spirulina*.

31. The composition according to claim 29, wherein said water in step a) is used as 8-15 times as that of said dry powder.

32. The composition according to claim 31, wherein said water in step a) is used as 10 times as that of said dry powder.

15 33. The composition according to claim 29, wherein said step b) is conducted at a temperature of 80°C-95°C.

34. The composition according to claim 33, wherein said step b) is conducted at a temperature of 90°C.

20 35. The composition according to claim 29, wherein said liquid phase in step c) is adjusted to pH 3.8-4.2.

36. An immunoenhancing composition comprising a therapeutically effective amount of proteoglycan extract of algae and/or a pharmaceutically acceptable carrier, wherein the proteoglycan extract of algae is prepared by the following steps of:

25 a) dissolving a dry powder of blue-green algae in 5-20 times water by weight, and conducting cellular walls-breaking;

d) heating a solution obtained from step a) at 60°-100°C, and cooling to separate a liquid phase from the solution;

c) adjusting pH of said liquid phase to less than 7, and filtering; and

d) adjusting the filter to pH 7, concentrating, and drying if necessary.

30 37. The composition according to claim 36, wherein said blue-green algae is *Spirulina*.

38. The composition according to claim 36, wherein said water in step a) is used as 8-15 times as that of said dry powder.

35 39. The composition according to claim 38, wherein said water in step a) is used as 10 times as that of said dry powder.

40. The composition according to claim 36, wherein said step b) is conducted at a temperature of 80°C-95°C.

41. The composition according to claim 40, wherein said step b) is conducted at a temperature of 90°C.

40 42. The composition according to claim 36, wherein said liquid phase in step c) is adjusted to pH 3.8-4.2.

43. A dendrite-like-cell-activating composition comprising a therapeutically effective amount of proteoglycan extract of algae and/or a pharmaceutically acceptable carrier, wherein the proteoglycan extract of algae is prepared by the

following steps of:

- a) dissolving a dry powder of blue-green algae in 5-20 times water by weight, and conducting cellular walls-breaking;
- b) heating a solution obtained from step a) at 60°-100°C, and cooling to separate a liquid phase from the solution;
- c) adjusting pH of said liquid phase to less than 7, and filtering; and
- d) adjusting the filter to pH 7, concentrating, and drying if necessary.

44. The composition according to claim 40, wherein said blue-green algae is *Spirulina*.

45. The composition according to claim 40, wherein said water in step a) is used as 8-15 times as that of said dry powder.

46. The composition according to claim 38, wherein said water in step a) is used as 10 times as that of said dry powder.

47. The composition according to claim 36, wherein said step b) is conducted at a temperature of 80°C-95°C.

48. The composition according to claim 37, wherein said step b) is conducted at a temperature of 90°C.

49. The composition according to claim 36, wherein said liquid phase in step c) is adjusted to pH 3.8-4.2.

50. A process for preparing a proteoglycan extract of algae, including the following steps of:

- a) dissolving a dry powder of blue-green algae in 5-20 times water by weight, and conducting cellular walls-breaking;
- b) heating a solution obtained from step a) at 60°-100°C, and cooling to separate a liquid phase from the solution;
- c) adjusting pH of said liquid phase to less than 7, and filtering; and
- d) adjusting the filter to pH 7, concentrating, and drying if necessary.

51. The process according to claim 50, wherein said blue-green algae is *Spirulina*.

52. The process according to claim 50, wherein said water in step a) is used as 8-15 times as that of said dry powder.

53. The composition according to claim 52, wherein said water in step a) is used as 10 times as that of said dry powder.

54. The process according to claim 50, wherein said step b) is conducted at a temperature of 80°C-95°C.

55. The composition according to claim 54, wherein said step b) is conducted at a temperature of 90°C.

56. The composition according to claim 50, wherein said liquid phase in step c) is adjusted to pH 3.8-4.2.



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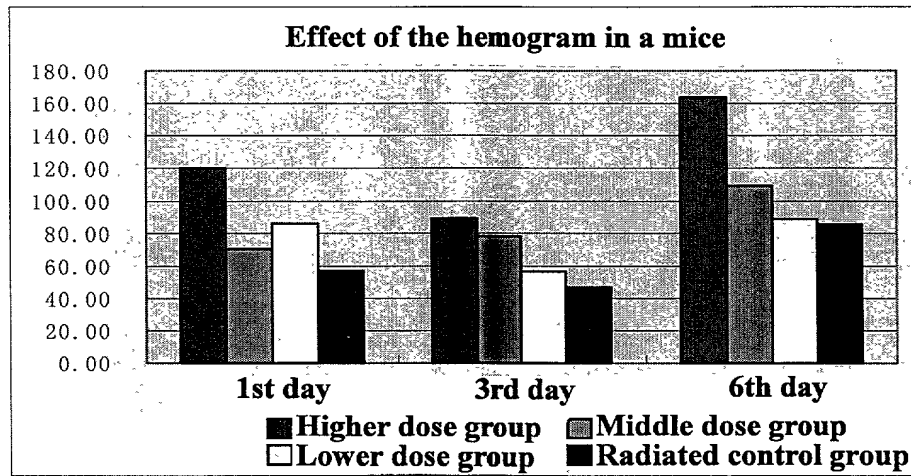


Fig. 1

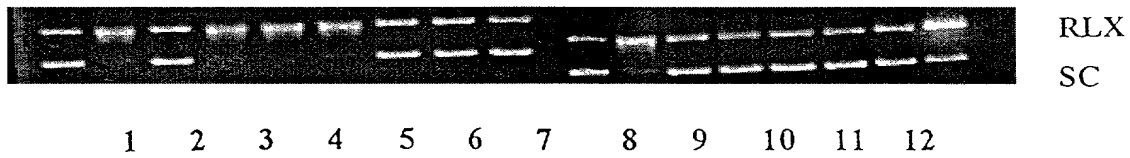


Fig. 2

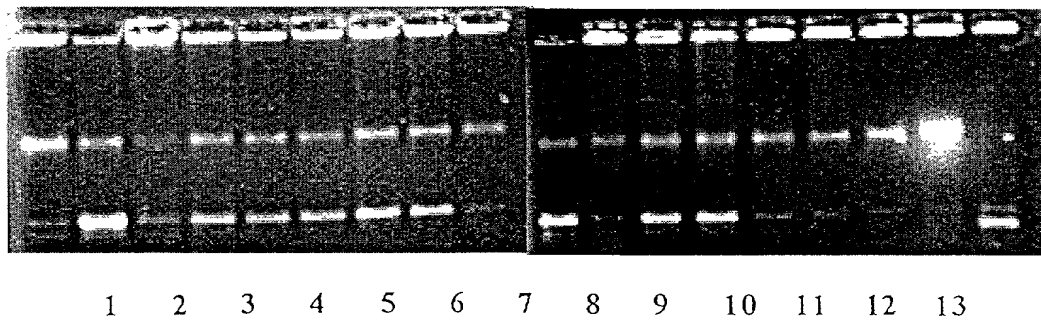


Fig. 3

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Fig. 4

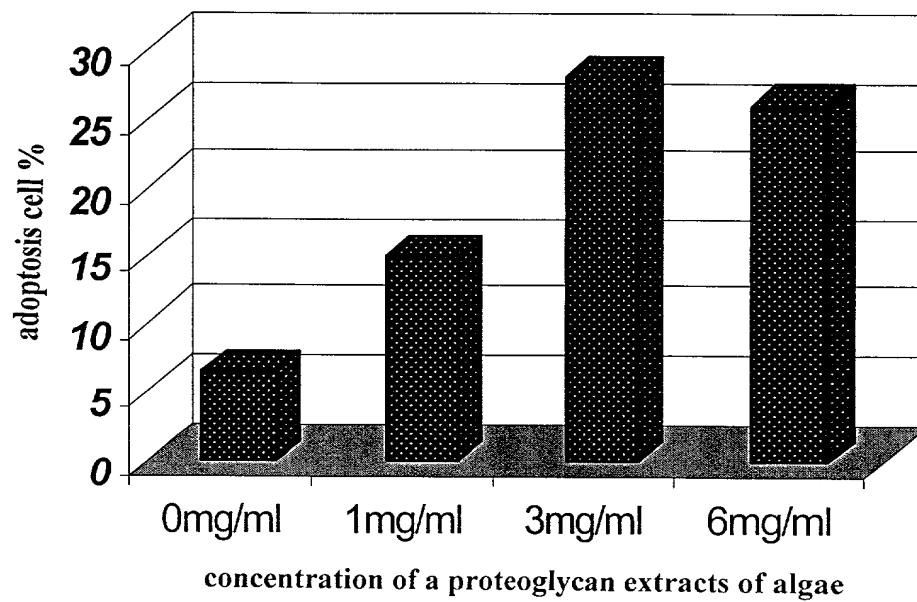


Fig. 5

Effect of different composition on cells of bone marrow in a mice

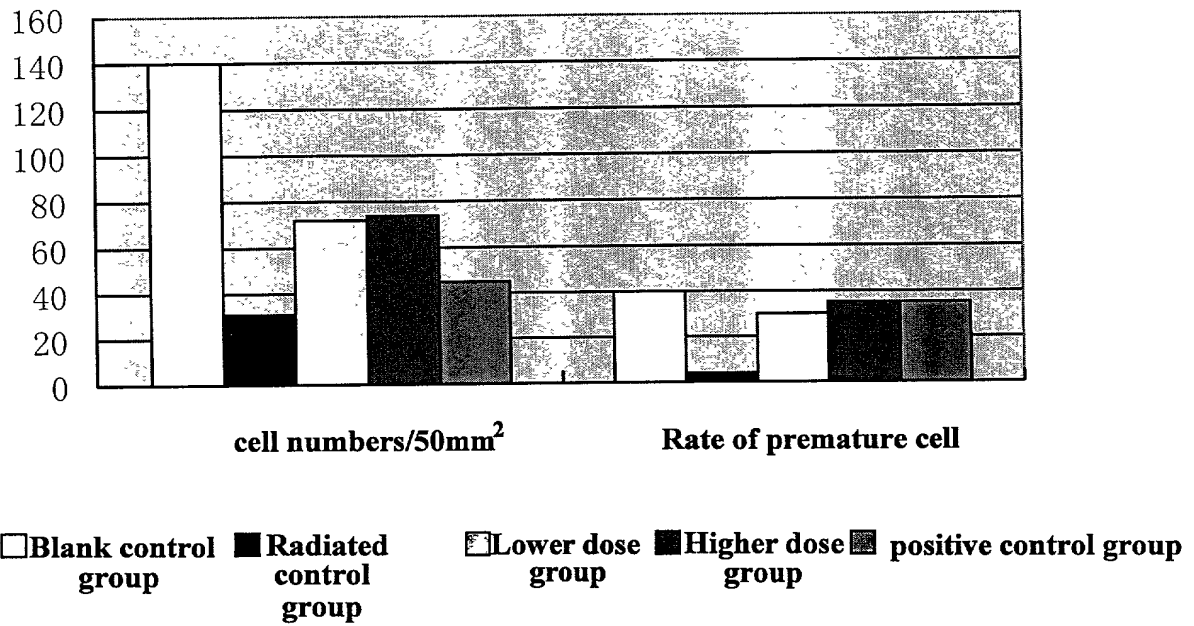


Fig. 6

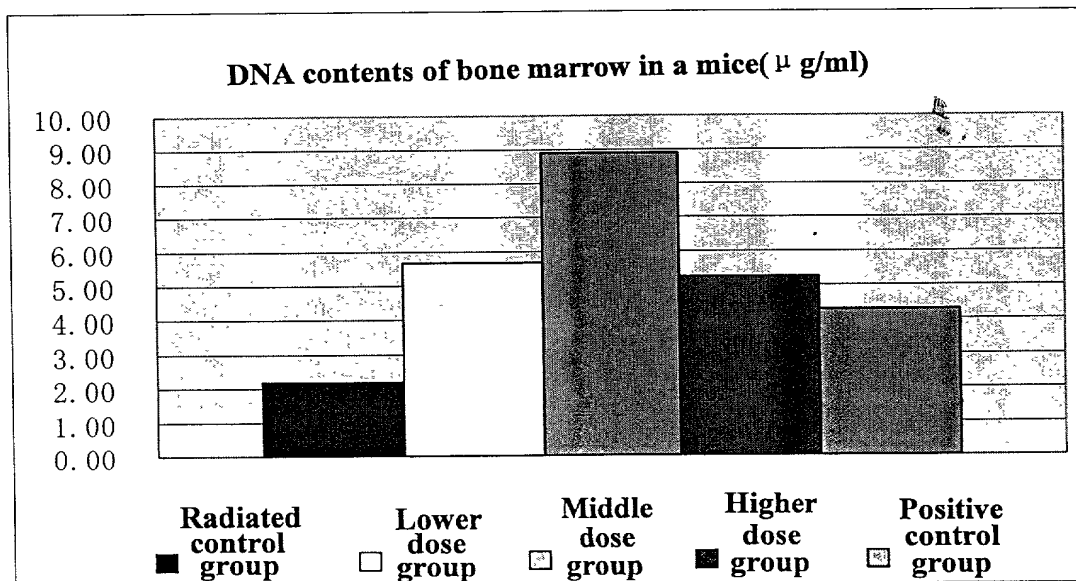


Fig. 7

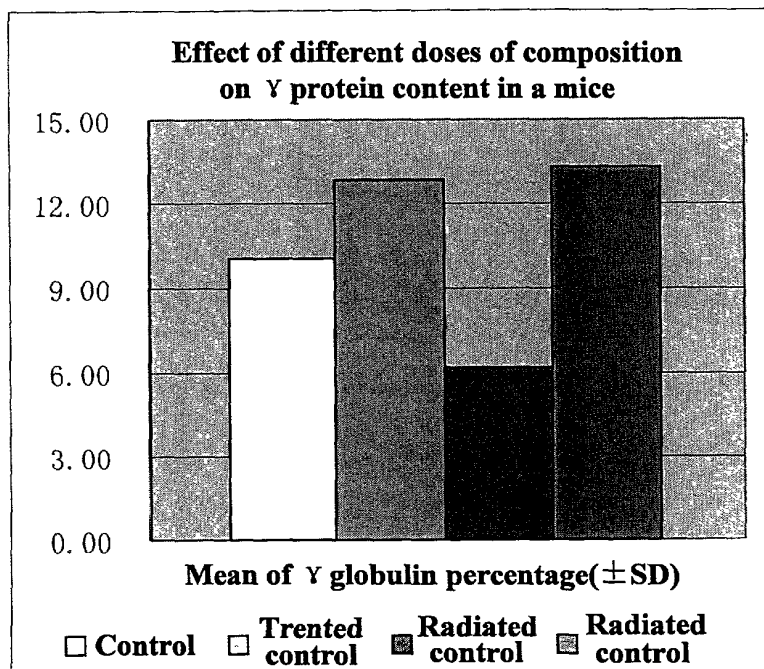


Fig. 8

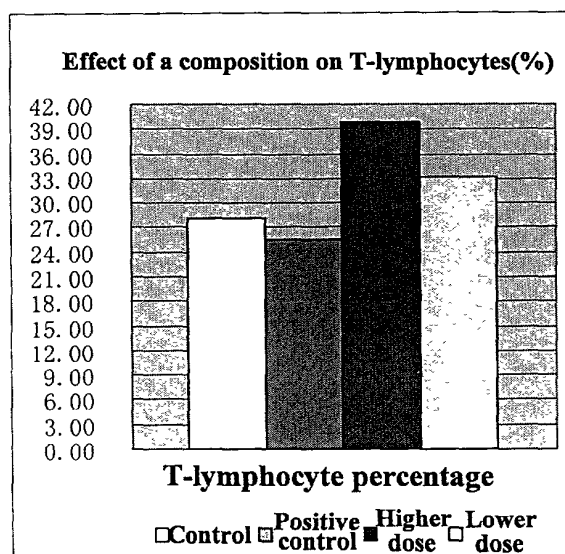


Fig. 9

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<b>DECLARATION FOR UTILITY OR DESIGN PATENT APPLICATION (37 CFR 1.63)</b>  <input checked="" type="checkbox"/> Declaration Submitted with Initial Filing      OR <input type="checkbox"/> Declaration Submitted after Initial Filing (surcharge (37 CFR 1.16 (e)) required)	<b>Attorney Docket Number</b>	CSI3-PT001
	<b>First Named Inventor</b>	Qi et al.
	<b>COMPLETE IF KNOWN</b>	
	<b>Application Number</b>	
	<b>Filing Date</b>	
	<b>Group Art Unit</b>	
	<b>Examiner Name</b>	

As a below named inventor, I hereby declare that:

My residence, post office address, and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

Proteoglycan Extracts of Algae and Uses thereof

the specification of which

(Title of the Invention)

☒ is attached hereto  
OR

☐ was filed on (MM/DD/YYYY)

as United States Application Number or PCT International

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I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment specifically referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR 1.56.

I hereby claim foreign priority benefits under 35 U.S.C. 119(a)-(d) or 365(b) of any foreign application(s) for patent or inventor's certificate, or 365(a) of any PCT international application which designated at least one country other than the United States of America, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or of any PCT international application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application Number(s)	Country	Foreign Filing Date (MM/DD/YYYY)	Priority Not Claimed	Certified Copy Attached?	
99109881.1	China	July 19, 1999	<input type="checkbox"/>	YES	NO
			<input type="checkbox"/>	<input type="checkbox"/>	<input checked="" type="checkbox"/>
			<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
			<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>

☐ Additional foreign application numbers are listed on a supplemental priority data sheet PTO/SB/02B attached hereto:

I hereby claim the benefit under 35 U.S.C. 119(e) of any United States provisional application(s) listed below.

Application Number(s)	Filing Date (MM/DD/YYYY)	
		<input type="checkbox"/> Additional provisional application numbers are listed on a supplemental priority data sheet PTO/SB/02B attached hereto.

[Page 1 of 2]

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## DECLARATION — Utility or Design Patent Application

I hereby claim the benefit under 35 U.S.C. 120 of any United States application(s), or 365(c) of any PCT international application designating the United States of America, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT international application in the manner provided by the first paragraph of 35 U.S.C. 112, I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR 1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application.

U.S. Parent Application or PCT Parent Number	Parent Filing Date (MM/DD/YYYY)	Parent Patent Number (if applicable)

☐ Additional U.S. or PCT international application numbers are listed on a supplemental priority data sheet PTO/SB/02B attached hereto.

As a named inventor, I hereby appoint the following registered practitioner(s) to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith:

Name	Registration Number	Name	Registration Number
Namely, the Attorneys of Volpe and Koenig, P.C.			

☐ Additional registered practitioner(s) named on supplemental Registered Practitioner Information sheet PTO/SB/02C attached hereto.

Direct all correspondence to: ☒ Customer Number 3624 OR ☐ Correspondence address below

Name	VOLPE AND KOENIG, P.C.		
Address			
Address			
City	State	ZIP	
Country	Telephone	Fax	

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. 1001 and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Name of Sole or First Inventor:		<input type="checkbox"/> A petition has been filed for this unsigned inventor	
Given Name (first and middle (if any))		Family Name or Surname	
Qing		Qi	
Inventor's Signature	Date		Jan. 15, 2002
Residence: City	Beijing	State	CNX
		Country	China
Citizenship	Chinese		
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Post Office Address			
City	Beijing	State	
		ZIP	100088
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☐ Additional inventors are being named on the \_\_\_\_\_ supplemental Additional Inventor(s) sheet(s) PTO/SB/02A attached hereto

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## DECLARATION

**ADDITIONAL INVENTOR(S)**  
**Supplemental Sheet**  
Page \_\_\_\_ of \_\_\_\_

**Name of Additional Joint Inventor, if any:**

☐ A petition has been filed for this unsigned inventor

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Family Name or Surname

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Ding

**Inventor's  
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**Inventor's  
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**Date**

**Residence: City**

**State**

**Country**

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**City**

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**ZIP**

**Country**

**Name of Additional Joint Inventor, if any:**

☐ A petition has been filed for this unsigned inventor

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**Inventor's  
Signature**

**Date**

**Residence: City**

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